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Immunization with the Vole Acid Fast Bacillus Against Experimental Tuberculosis. Wallace S. Brooke, Ph.D., M.D., and Robert Day, M.D. 275

Favism. Hugh W. Josephs 295

Experimental Studies on Sulfapyrazine in Mice. Gordon I. Trevett 299

Observations on a Diet Deficient in Both Methionine and Cystine in Man. Anthony A. Albanese, L. Emmett Holt, Jr., Joseph E. Brumback, Jr., Jane E. Frankston and Virginia Irby 308

Proceedings of the Meeting of the Johns Hopkins Medical Society. Held in Hurd Hall, April 10, 1944. Dr. Alfred Blalock 313

Book Reviews 316

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IMMUNIZATION WITH THE VOLE ACID FAST BACILLUS AGAINST EXPERIMENTAL TUBERCULOSIS

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From the Department of Medicine of the Johns Hopkins Medical School and the Johns Hopkins Hospital

TABLE OF CONTENTS

Introduction 275
Experimental 277
I. Description of Animal Groups and the Method of Vaccination and Infection 277
II. Status of Vaccinated Animals before Infection 279
A. Viable Vole Vaccinated Animals 279
B. Animals Vaccinated with Heat-killed Vole Acid-fast Bacilli 279
C. Animals Vaccinated with B.C.G. 280
III. Results of Experimental Infection 280
A. Gross Pathology 280
B. Microscopic Pathology 282
C. Tuberculin Sensitivity 282
Discussion 284
Conclusions 287
Bibliography 288

INTRODUCTION

Since the original report of the significant immunity against experimental human tuberculosis induced by the vole bacillus in the guinea pig, Wells and Brooke (1940), it has been shown by Griffith and Dalling (1940) that the calf is protected against the bovine tubercle bacillus. Large scale experiments are being conducted now in England with the hope that the vole acid-fast bacillus will prove useful against tuberculosis in bovine animals. Since approximately 35 per cent of English and Continental dairy herds react positively to tuberculin, a safe vaccine should be of tremendous practical value.

The vole bacillus is a relatively recent bacteriological discovery, isolated by Wells in 1937. In 1940 Brooke published his results of a two year study of its properties. This bacillus is an apparently stable, slow-growing, waxy Mycobactium, which is more pleomorphic in tissue than the human and bovine tubercle bacillus, but cannot be distin-

guished from them by serological absorption of agglutinins. It produces a tuberculin which gives allergic skin reactions in animals infected with mammalian tubercle bacilli.

The vole acid-fast bacillus does not appear to be an attenuated bovine, human, or avian bacillus, nor a form of the murine or human leprosy bacillus. Its distinguishing characteristics seem to be fixed. This particular organism is apparently (Brooke, 1940–1941) a third type of fixed mammalian tubercle bacillus, i.e. in addition to the human and bovine types. In some properties it may be a "link" between tubercle and leprosy mycobacteria.

According to Wells, Brooke, and Griffith (1939, 1940, 1941) the vole bacillus is highly pathogenic only for the vole. In this animal it produces a very slowly progressive disease with subcutaneous lesions paramount—like those of rat leprosy in some respects. The vole bacillus causes localized lesions in cattle, hamsters, guinea pigs, rabbits, and white rats without progressive systemic disease, unless overwhelming doses are used. It is innocuous to the fowl.

The rabbit is somewhat protected from bovine tuberculosis by vaccination with the vole acid-fast bacillus (Brooke, 1940), although the extent and nature of the immunity require further study.

There are as yet no published accounts of its pathogenicity for monkey or man. Our preliminary, unpublished work indicates that progressive vole bacillus infection in monkey and man is improbable. One mgm. (moist weight) of viable vole bacilli given subcutaneously and intravenously in the human has given no evidence that it will produce progressive disease.

The vole acid-fast bacillus has been used by us in a few Macacus rhesus monkeys as an immunizing agent against human type tuberculosis. The results, to be published soon, are encouraging.

The vole acid-fast bacillus was chosen here as an immunizing agent because we believe it to be avirulent for guinea pigs and rabbits, and to be virulent only for the vole among other laboratory animals, Brooke (1941). It has already given evidence of inducing marked protection against experimental tuberculosis in guinea pigs and cattle. (Wells and Brooke, 1940 and J. A. M. A. ed. 1941). Additional reasons for its use as a vaccine against the disease caused by the human tubercle bacillus are, (a) that it can not be differentiated serologically from the human tubercle bacillus and (b) that it forms

specific products in the culture medium which give allergic skin reactions in animals infected with human tuberculosis, (Brooke 1940). These properties indicate antigenic and metabolic similarity to the human tubercle bacillus and one might expect, therefore, a higher immunizing capacity than if such properties did not exist.

This paper is a further report on the degree of immunity in guinea pigs vaccinated with viable and heat-killed vole acid-fast bacilli to subsequent infection with virulent human tubercle bacilli. Because the Bacillus Calmette-Guerin (B.C.G.) has been very widely used and apparently induces more protection than other avirulent mycobacteria hitherto reported, a comparable group of guinea pigs vaccinated with B.C.G. was studied.

EXPERIMENTAL

I. Description of Animal Groups and the Method of Vaccination and Infection

One hundred and fifty-seven guinea pigs of approximately the same age and weight and obtained from the same breeder were used in these experiments. These animals were in good condition and were tuberculin-negative before vaccination. Throughout the sixteen months of the experiment they were housed in cages containing four to seven guinea pigs of the same group and sex, and there was no interchange of population.

Group I. There were 43 animals in this group which were vaccinated with 4 mgms. (moist weight) of *viable vole acid-fast bacilli* seven weeks before infection with human tubercle bacilli, and again with 1 mgm. of vole bacilli three weeks before infection. All vaccinations were in the subcutaneous tissues of the groin.

The vaccine was prepared by carefully weighing moist scrapings of eight week cultures and grinding them in an agate mortar for one-half an hour to obtain a homogeneous suspension when diluted in normal saline solution.

Group II. There were 41 animals in this group which were treated exactly like those of Group I except that vaccinations were with equivalent amounts of *heat-killed vole acid-fast bacilli* instead of with viable bacilli. The thermal death point was assured by heating to 65° for thirty minutes.

Group III. There were 41 animals in this group. B.C.G. was used

as the immunizing agent, and they were given 10 mgm. (moist weight) seven weeks before infection and 10 mgm. again three weeks before infection.[1]

Group IV. There were 32 animals in this group which were used as *controls*.

Seven weeks after the first vaccination date, four animals were sacrificed and the remaining 153 were given an infecting dose of a recently isolated strain of virulent human tubercle bacillus. This strain was obtained from a guinea pig which had been inoculated with sputum from a patient at the Johns Hopkins Hospital who had advanced pulmonary tuberculosis.

A four weeks' culture of this bacillus on Petragnini's medium was scraped and weighed carefully to the fourth decimal place. The suspension was prepared from the homogeneous material obtained by grinding the weighed growth for one hour in a smooth agate mortar. Dilution was made so that 0.1 cc. equaled 0.000,001 mgm. of moist weight. All guinea pigs were given this dose subcutaneously in the right axilla. (Vaccinations had been given in the inguinal region).

The infecting dose used in our experiments was planned to equal about 1000 total organisms, of which 100 were assumed to be viable. According to Wilson and Schwabacher (1937) there is good evidence to suggest that the total number of tubercle bacilli alive and dead contained in 1 mgm. of moist weight of growth from a two to three weeks' old culture on glycerine egg medium is roughly 1,000 to 1,500 million. The viability of such a growth of human bacillus, as determined by the same authors, is probably about 5 to 10 per cent. Thus in 0.000,001 mgm. there should be roughly 100 viable bacilli.

It must be stressed that so small an infecting inoculum was selected for definite, and, we believe, important reasons. Doerr and Gold (1932), and others, have stated that the subcutaneous injection of a single virulent mammalian tubercle bacillus may succeed in producing a slow type of disease without generalization. Inoculation of between 10 and 100 bacilli usually causes a slowly progressive disease, and if larger numbers of bacilli are introduced, death occurs in from six to twenty weeks.

[1] Different vaccinating doses of B.C.G. (10 mgms. and 10 mgms.) and of the vole bacillus (4 mgms. and 1 mgm.) were chosen arbitrarily.

We believe that it is unwise to attempt to measure immunity against experimental tuberculosis when millions or billions of virulent infecting bacilli are given subcutaneously. These huge infecting doses used by some investigators will not bring out small or even moderate differences between immunizing agents and must be viewed as gross and overwhelming insults to the animal organism. In almost no case is the human being submitted to such dosages when he becomes infected, for at the most he receives hundreds or thousands of bacilli, usually intrapharyngeally, and, of course, not all of these gain access to tissues.

In addition, immunity against tuberculosis is such that it can be overridden with heavy inocula of virulent bacilli. If one does not encounter such large infecting doses clinically in so resistant a host as man, it is unwarranted to use experimental techniques which overpower the relatively weak natural immunity of the guinea pig.

Suffice it to say that whatever the error inherent in progressive dilutions from a homogeneous suspension, progressive generalized disease occurred in all of our control guinea pigs.

II. Status of Vaccinated Animals Before Infection

A. Animals Vaccinated with Viable Vole Bacilli. Within two to three weeks after vaccinating a guinea pig in the groin, a firm circumscribed nodule could usually be palpated in the injection site. Ulceration almost invariably occurred within two to five weeks, with a discharge of heavy creamy pus.

Seven weeks after vaccination two animals of this group were sacrificed and showed no gross or microscopic lesions typical of acid-fast bacillary infection except for small circumscribed sacs of caseation at the site of vaccination with the vole bacillus. These sacs were quite typical of a 7 weeks' vole bacillus infection in the guinea pig. The local ulcerations which appeared earlier usually healed by the end of seven weeks, but the subcutaneous or subfascial caseation often took months to disappear. [See Plates I and II.]

B. Animals Vaccinated with Heat-killed Vole Acid-fast Bacilli. These animals did not differ significantly in gross appearance or in microscopic reaction from those injected with the viable bacilli. Ulceration occurred at approximately the same time with the discharge of about the same amount of pus. One animal of this group was sacri-

ficed at the end of seven weeks. The animal looked the same at necropsy as did those vaccinated with the live bacilli.

C. Animals Vaccinated with B.C.G. Before infection there was nothing to differentiate animals vaccinated with B.C.G. from those vaccinated with the vole bacillus, except that the gross reaction of the former to vaccination (local caseation with ulceration and subsequent healing) showed a more rapid tendency to heal than did the latter. This result need not be viewed as constant; it was not marked and may well vary with different strains of B.C.G. and of the vole bacillus.

One animal in this group was sacrificed 7 weeks after the first vaccination. It failed to show any gross or microscopic lesions typical of tuberculosis other than a small local (inguinal) caseous abscess.

III. Results of Experimental Infections

Animals vaccinated with viable vole acid-fast bacilli will be referred to as "V.V."; animals vaccinated with B.C.G. as "B.C.G."; and animals vaccinated with heat-killed vole vaccine as "H.K.V.V."

Seven weeks after the first and three weeks after the second vaccination all vaccinated and unvaccinated animals were infected, as described above, with 0.000,001 mgm. of a freshly isolated growth of human tubercle bacilli. The results of infection were judged in three ways:

1. By the study of gross pathology.
2. By the study of microscopic pathology.
3. By periodic tuberculin testing of the infected animals.

A. Gross Pathology. During the 14 months subsequent to infection all dead animals were examined and placed, as follows, in one of three pathological groups, according to the degree of gross tuberculous infection found at necropsy:

No gross Tbc.: No gross tuberculosis seen.

Minimal Tbc.: One organ only (spleen, liver or lung) grossly infected with or without microscopic lymph node involvement.

Generalized Tbc.: Two or more organs grossly infected; or both lungs extensively infected.

Altogether 96 animals were sacrificed at planned time intervals (see Table 1). All other animals died either of tuberculosis or of intercurrent disease. The experiment was completed in 14 months.

In summary of the gross tuberculosis found at necropsy it can be said:

1. All controls alive after 4 months developed generalized tuberculosis. At 9 months 78 per cent, and at 14 months 88 per cent had died with generalized tuberculosis.

TABLE 1

Total deaths according to degree of gross tuberculosis, and cumulative percentages of deaths in each experimental group from 0 to 14 months after infection with virulent human tubercle bacilli

DEGREE OF TUBERCULOSIS	TYPE OF INOCULATION	0–3 MONTHS		4–6 MONTHS		7–9 MONTHS		10–12 MONTHS		13–14 MONTHS		0–14 MONTHS
		No. of deaths	Cumulative per cent of all deaths	No. of deaths	Cumulative per cent of all deaths	No. of deaths	Cumulative per cent of all deaths	No. of deaths	Cumulative per cent of all deaths	No. of deaths	Cumulative per cent of all deaths	Total no. of deaths
Tbc. +++	V.V.	0	0	0	0	3	7.3	2	12.2	3	19.5	8
	H.K.V.V.	0	0	12	30.0	2	35.0	2	40.0	0	40.0	16
	B.C.G.	0	0	3	7.5	1	10.0	2	15.0	1	17.5	7
	Control	2	6.3	20	68.9	3	78.3	3	87.7	0	87.7	28
Tbc. ++	V.V.	0	0	3	7.3	0	7.3	0	7.3	1	9.7	4
	H.K.V.V.	1	2.5	3	10.0	0	10.0	0	10.0	1	12.5	5
	B.C.G.	0	0	6	15.0	4	25.0	1	27.5	2	32.5	13
	Control	2	6.3	0	6.3	0	6.3	0	6.3	0	6.3	2
No gross Tbc.	V.V.	2	4.9	10	29.3	2	34.2	2	39.1	13	70.8	29
	H.K.V.V.	3	7.5	4	17.5	5	30.0	0	30.0	7	47.5	19
	B.C.G.	2	5.0	11	32.5	4	42.5	3	50.0	0	50.0	20
	Control	0	0	2	6.3	0	6.3	0	6.3	0	6.3	2
All groups	V.V.	2	4.9	13	36.6	5	48.8	4	58.6	17	100	41
	H.K.V.V.	4	10.0	19	57.5	7	75.0	2	80.0	8	100	40
	B.C.G.	2	5.0	20	55.0	9	77.5	6	92.5	3	100	40
	Control	4	12.6	22	81.5	3	91.2	3	100	0	100	32

+++ Tbc. Generalized tuberculosis: a) Two or more organs grossly infected; or b) Both lungs extensively involved.

++ TBc. One organ only (spleen, liver, or lung), grossly infected with or without microscopic lymph node involvement.

2. Of the HKVV animals, at 9 months, 35 per cent had died with generalized Tbc. At 14 months 40 per cent had died with generalized Tbc., but 48 per cent had shown no gross tuberculosis at necropsy.

3. Of the VV and BCG animals, at 9 months roughly 10 per cent, and at 14 months roughly 18 per cent of each group were dead with generalized Tbc. *At 14 months 71 per cent of all VV and 50 per cent*

of all BCG animals had been free of gross tuberculosis at the time of death. The difference between the VV and BCG groups may be a significant one.[2] Further evidence must be adduced, however, before it can be stated that the D-15 viable vole bacillus of this experiment is superior to BCG in protecting guinea pigs against experimental human tuberculosis.

B. Microscopic Pathology. 88 out of 153 guinea pigs were examined by microscopic study of the following tissues: 1) axillary or tracheobronchial lymph node, 2) lung, 3) liver, 4) spleen, 5) kidney. The blocks were representative of the organs from which they were taken and were stained after formol Zenker fixation with hematoxylin and eosin.

The microscopic sections in general bore out the gross impression.[3] It was not possible to say, however, from their study that any differences in protective power existed between the BCG and the viable vole vaccine used in the present experiment.

TABLE 1a

Percentage of Guinea Pigs in each Group Dying with Generalized Tuberculosis

	HKVV	VV	BCG	CONTROL
1 to 6 mos.	30	0	7.5	69
1 to 14 mos.	40	19.5	17.5	88

C. Tuberculin Sensitivity. Animals were periodically tested intracutaneously in the shaven abdominal skin with 0.1 cc. of Old Tuberculin in dilutions of 1/100, 1/1000. 1/10000, and 1/100000 (1 mgm., 0.1 mgm., 0.01 mgm., and 0.001 mgm. O.T.).

Reactions were read at 48 hours as follows:

Negative: No reaction.

?	0–5 mm. erythema
+	5 to 10 mm. erythema ± induration
++	10 to 20 mm. erythema ± induration
+++	More than 20 mm. erythema ± induration
++++	Necrosis and erythema

[2] Figures analyzed by Mr. H. M. C. Luykx of the Department of Biostatistics, Johns Hopkins School of Public Health.

[3] Occasional discrepancy between the gross and microscopic findings was due to the failure to take sufficiently large tissue blocks and to technical errors in preparation.

In tabulating results only positive reactions (5 mm. erythema or more) were considered (table 3).

1. *Tuberculin Sensitivity after Vaccination and before Infection.* Animals were tested both before vaccination and 2 weeks, 4 weeks, and

TABLE 2

Gross Tuberculosis, Microscopic Tuberculosis, and Antemortem (less than 72 hours) Tuberculin Sensitivity in 88 Guinea Pigs

V.V.				HKVV				BCG				CONTROL			
	Gross Tbc.	Micro. Tbc.	Tuberculin		Gross Tbc.	Micro. Tbc.	Tuberculin		Gross Tbc.	Micro. Tbc.	Tuberculin		Gross Tbc.	Micro. Tbc.	Tuberculin
1	0	0	2	1	0	0	2	1	2+	0	3	1	3+	3+	—
2	0	0	3	2	0	0	3	2	0	Spleen	2	2	3+	3+	4
3	3+	3+	0	3	0	0	—	3	0	0	—	3	2+	Spleen	3
4	0	0	2	4	0	0	3	4	3+	0	3	4	3+	3+	4
5	0	0	3	5	0	0	3	5	2+	0	2	5	3+	3+	4
6	0	0	3	6	3+	3+	4	6	2+	0	3	6	3+	3+	4
7	0	0	3	7	0	0	4	7	0	0	3	7	3+	3+	4
8	2+	Spleen	3	8	2+	0	2	8	2+	0	2	8	2+	3+	—
9	2+	0	3	9	0	0	3	9	2+	0	2	9	3+	Spleen	—
10	3+	3+	4	10	0	0	—	10	0	0	2	10	3+	3+	—
11	0	0	2	11	2+	0	0	11	2+	0	2	11	3+	3+	—
12	0	0	2	12	3+	Spleen	2	12	0	0	0	12	3+	3+	—
13	0	0	2	13	3+	3+	4	13	3+	3+	—	13	3+	3+	3
14	3+	Spleen	0	14	3+	Spleen	3	14	0	T. B. gland	2	14	3+	3+	4
15	0	0	2	15	2+	0	0	15	2+	T. B. gland	—	15	3+	3+	3
16	0	0	3	16	0	0	3	16	2+	0	3	16	3+	3+	—
17	0	Ax. gland	3	17	0	0	2	17	0	0	3	17	3+	3+	—
18	0	0	?2	18	2+	T. B. gland	—	18	0	T. B. gland	—	18	3+	3+	—
19	0	0	2	19	0	0	2	19	2+	0	—				
20	2+	0	2	20	0	0	2	20	2+	T. B. gland	—				
21	0	0	—	21	0	0	2								
22	0	0	2												
23	2+	0	?2												
24	0	0	2												
25	0	0	—												
26	0	0	—												
27	0	0	2												
28	0	0	3												
29	3+	3+	3												

Key to tuberculin sensitivity: 0, insensitive to 1 mgm.; 2, sensitive to 1 mgm.; 3, sensitive to 0.1 mgm.; 4, sensitive to 0.01 mgm.

Key to gross pathology: 0, no gross tuberculosis; 2+, minimal tuberculosis; 3+, generalized tuberculosis.

7 weeks after vaccination. There was no significant difference in sensitivity to Old Tuberculin between the vaccinated groups during the period before infection with human tuberculosis. Seven weeks after vaccination all animals tested (six in each vaccinated group)

were sensitive to 1.0 mgm. O.T. but not to 0.1 mgm. O.T. The reactions conform with results previously reported by Brooke (1941).

2. *Tuberculin Sensitivity after Infection with Human Tubercle Bacilli.* Animals were tested 2 months, 3 months, 4 months and 14 months after infection. Differences began to appear at 3 months (table 3).

In general, the greatest tuberculin sensitivity accompanied the greatest degree of tuberculous infection. Only one animal out of 20 sensitive to 0.01 mgm. at 4 months failed to develop gross and microscopic evidence of generalized tuberculosis (table 3).

TABLE 3

Highest Tuberculin Dilutions to Which Guinea Pigs Reacted Positively, When Tested 3 Months, 4 Months, and 14 Months After Infection with Human Tubercle Bacilli

TUBERCULIN SENSITIVITY	3 MONTHS				4 MONTHS				14 MONTHS			
	VV	HKVV	BCG	Control	VV	HKVV	BCG	Control	VV	HKVV	BCG	Control
Not sensitive to 1 mg.....	0	0	0	0	6	1	0	0	4	1	0	All dead
Sensitive to 1 mg.....	10	9	9	5	18	4	18	1	5	2	1	
Sensitive to 0.1 mg.....	0	1	1	6	14	10	20	2	6	2	1	
Sensitive to 0.01 mg.....	0	0	0	0	0	6	0	14	1	0	0	
Number tested.....	10	10	10	11	38	21	38	17	16	5	2	0

In the lower dilutions (1 mg. and 0.1 mg. O.T.) the correlation, if any, between infection and sensitivity was not obvious.

No significant differences in tuberculin sensitivity existed between the VV and BCG groups at any stage of the experiment. The control animals developed the highest degree of sensitivity. The HKVV animals were somewhat less sensitive than the controls but more so than the VV and BCG animals.

DISCUSSION

In the present experiment the vole acid fast bacillus conferred a significant resistance on guinea pigs against experimental human

tuberculosis without itself producing serious disease. Not only was the onset of gross infection delayed, but its severity during the 14 months of the experiment was diminished.

This diminution in severity, however, was not uniform. The protected animals were relatively well-protected, but most of the grossly tuberculous animals were extensively infected. The resistance must be evaluated cautiously, because of the peculiar characteristics of the tubercle bacillus in the animal host. Immunity in tuberculosis is rarely, if ever, complete and any infection must be considered potentially dangerous, whatever the resistant state of the animal.

In the present experiments no statistically significant differences between the VV and the BCG animals could be demonstrated on the basis of gross and microscopic pathology or of tuberculin sensitivity after infection, although the VV animals seemed better protected in that severe infection was somewhat more delayed and gross tuberculous involvement slighter than in the BCG group. It is to be remembered that the vaccinating dose of BCG (20 mgms.) was four times that of the vole bacillus (5 mgms.). If equal doses had been given, perhaps this differential immunity would have been more striking.

Tuberculin testing after infection failed to bring out real differences between the two groups.

This paper is the second report of work by one of us (W. S. B.) designed to compare the immunizing power of the vole vaccine with that of viable BCG, the first experiments having been those of Wells and Brooke in 1940. In the earlier experiment the animals were vaccinated subcutaneously with 0.5 mg. of the vole D-15 strain or 7.5 mg. of BCG at weekly intervals for one month and were infected with 0.000001 mg. of a virulent human strain 5 months after the last vaccination.

In the present experiment the animals were vaccinated with 4 mg. of the vole D-15 or 10 mg. of BCG, 7 weeks before infection, and with 1 mg. of D-15, or 10 mg. of BCG, 3 weeks before infection with 0.000,-001 mgm. of a virulent human strain. In both experiments the BCG animals were given larger vaccination doses than were the vole animals. We have no evidence to show that this amount of B.C.G. was primarily "toxic" to our guinea pigs.

In the former experiment all surviving animals were killed at 11

weeks; in this experiment a large number of animals were killed 16 weeks after infection. (Plate III). The lesions in the VV animals of both experiments were minimal; the BCG animals in the 1940 experiment were riddled with tuberculosis, but in the present experiment were almost as well protected as the VV guinea pigs.

The discrepancy in results is explained by assuming either (a) that a more effective strain of BCG was used in the present experiment and/or (b) that a less effective strain of the viable vole bacillus was used in the present experiment and/or (c) that the 5 month interval between vaccination and infection in the former experiment was sufficient to allow the BCG produced immunity to lapse, and was not sufficient to allow the immunity produced by the vole bacillus to lapse. At any rate the VV animals were relatively well protected in both experiments. The BCG vaccine seems to have been the variable factor.[4]

Irwin and O'Connell (1943) report that both living and heat-killed vole bacilli were effective in producing resistance against 0.0001 mgm. of human tubercle bacilli in the guinea pig. Each animal received a weekly dose of 0.5 mgms. (2.0 mgms. in all) after four weekly subcutaneous doses of the LV 285 vole acid-fast strain. These authors found their control animals extensively infected 6 weeks after inoculation with the human strain, but their vaccinated guinea pigs, protected with either living or heat-killed vole vaccine, showed only minimal lesions at the same time.

Corper and Cohn (1943) report the results of vaccinating small, comparable numbers of guinea pigs with the vole bacillus, the avirulent human bacillus and B.C.G. The infecting dose of virulent human bacilli was 0.0001 mgm. All vaccinated animals were found to be much more resistant than the controls, but no significant difference existed between the vaccinated groups. They offer evidence that the D-15 strain of the vole acid-fast bacillus may produce progressive disease in itself when given intravenously in large amounts.

The work of Forkner and Shultz of the New York Hospital likewise

[4] In the original work of Wells and Brooke the BCG strain used was obtained from Dr. A. S. Griffith and sent to him from Calmette in 1921. It had gone through 260 generations, mostly subcultures upon bile-egg media. The BCG strain used in the present work is that used by the New York Board of Health; it was obtained from Dr. E. L. Opie.

indicates that the D-15 strain may produce in some labratory animals slowly progressive disease [personal communication].

Whether or not all strains of the vole bacillus cause progressive disease is an important consideration. However, guinea pigs given as much as 10 mgms. of strain G 202 subcutaneously, orally or into the conjunctivae have not died or shown signs of progressive disease after six months. In addition four guinea pigs given 2 mgms., 5 mgms., 10 mgms., and 15 mgms. of strain D-15 intravenously showed no gross tuberculous pathology at 10 weeks. The vole bacillus is not always innocuous when given intravenously. Occasional guinea pigs die of apparent vole infection after both intravenous and subcutaneous inoculation of 1 mgm. and 10 mgms. of certain vole strains (Brooke, 1940).

In view of the discrepancy between the virulence of early, recently isolated strains and later sub-cultured strains, it is possible that the vole bacillus is acquiring greater pathogenicity.

Many of the properties of the vole bacillus are as yet unknown, and a suitable medium is needed to preserve the low virulence of the bacillus in sub-culture, but a number of hopes are raised by its discovery. First of these is its potential use in the control of bovine tuberculosis in the British Isles (Dalling, 1942). Second, with avirulent strains, it carries promise, however remote, in the immunization of uninfected children and adults who are exposed to tuberculosis at home or at work. Last are the possibilities of its use in conjunction with chemotherapeutic agents, of which promin and diasone may be precursors, in the prevention and treatment of tuberculosis.

CONCLUSIONS

1. The living D-15 strain of the vole acid-fast bacillus was used to immunize guinea pigs against experimental human tuberculosis. It significantly delayed the onset and diminished the severity of infection.

2. Under the conditions of the experiment, the viable vole bacillus vaccine was as effective as B.C.G. against infection with virulent human tubercle bacilli. There is evidence to suggest that it may be superior to B.C.G.

3. A heat-killed vole bacillus vaccine showed a less, but significant, degree of immunizing power.

ACKNOWLEDGMENTS

We would express our appreciation for their encouragement and advice to Dr. Warfield T. Longcope and Dr. Arnold R. Rich. This study was made possible by a grant from the research fund of the Dept. of Medicine of the Johns Hopkins University and the Johns Hopkins Hospital.

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PLATE 1

Plate I shows the vole acid-fast bacillus in tissue *seven days after inoculation* of the bacillus into a guinea pig. There is not much cellular reaction, few, if any, true giant cells and minimal lymphocytic response. Curved, shepherd's crook and S-shaped forms exist typical of the vole bacillus in tissue. The animal is not sensitive to tuberculin at this date.

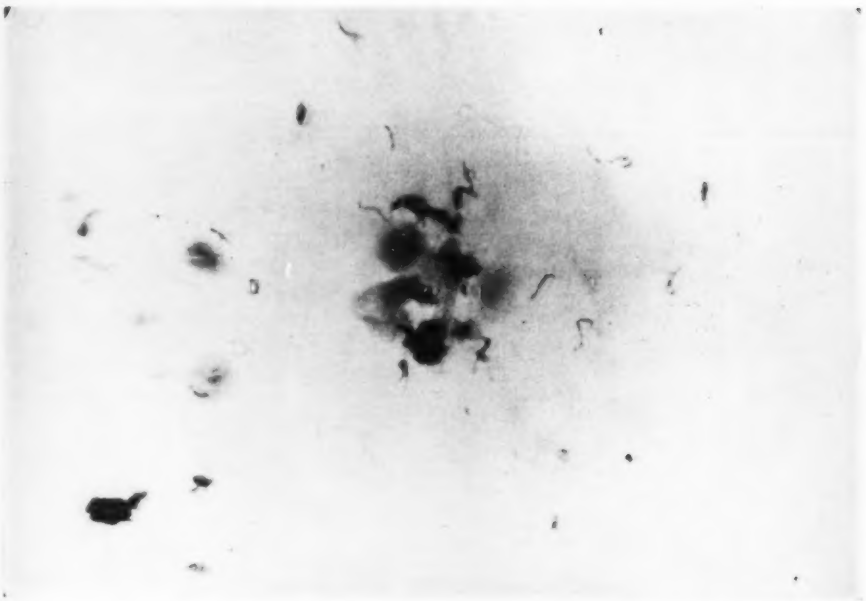

PLATE 2

This picture shows the characteristic reaction in the guinea pig *three weeks after infection* with four mgms. of the viable vole vaccine subcutaneously in the groin. There is local caseation with or without inguinal enlargement. The animal does not appear ill. This local condition may continue on for some months, usually subsiding slowly.

PLATE 3

Plate III shows the spleens of approximately one dozen guinea pigs of each group sacrificed four months after infection with virulent human tubercle bacilli. It can be seen that all spleens of the controls are extensively involved and that some of the heat-killed vole vaccinated spleens show gross tuberculosis. In the B.C.G. group the 5th and the 9th spleen show gross tubercles. The 1st spleen of the viable vole vaccinated group is enlarged without gross tuberculosis.

UNIV. OF MICH.

FAVISM

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Favism (1) may be defined as an acute hemolytic anemia resulting from contact with the fava bean or the plant from which it comes. The disease is common in Sardinia; it occurs with less frequency in Sicily and Southern Italy and is almost unknown elsewhere. Only twice has it been reported in this country (2, 3). It is the purpose of this report to describe a typical instance of the disease in a 5 year old boy observed in Baltimore, and to call attention to the fact that the fava bean, although not grown in this country, is not infrequently imported by immigrants from the Mediterranean littoral who have formed the habit of eating it in the home country. It appears likely that many more cases of favism would be discovered here if the condition were kept in mind.

CASE REPORT

A. K. (H.L.H. #-15145) a 5 year old boy of Greek parentage was brought to the Harriet Lane Home on March 16, 1940 with the complaint of jaundice, fever, abdominal pain and the passage of dark urine.

Family History. The father, 43, and the mother, 29, were in good health and gave no history of jaundice or anything suggesting hemolytic crises in themselves or their relatives. An older brother of the patient, 8 years of age, had had an attack of jaundice at 4 years lasting 4 days. No details are known except that it was unaccompanied by abdominal pain. In other respects he had been well. Fragility studies of the red cells and of this older brother and of the parents revealed normal values.

Past History. The patient had suffered from various respiratory infections. He had contracted measles and mumps at 2 years, and varicella at 3 years. Eleven months before admission he had developed an attack similar to the present one, lasting about 10 days.

Present Illness. 4 days before admission, having previously been well, the patient complained of severe abdominal pain which became worse when he voided. His urine was described as brown in color and his stools as dark. Several mild nosebleeds occurred during the 4 days prior to admission. Jaundice became conspicuous on the second day of the illness, but was thought to be less marked on the

day of admission. There had been some fever and the appetite had been poor, but was improving at the time of admission.

Physical Examination. Temp. 37.5°C. Pulse 120. Resp. 24. B.P. 85/50. The patient was well developed, but somewhat thin. Pallor was striking. There was an icteric tint to the skin and the sclerae were conspicuously jaundiced. The throat was not inflamed. The lungs were clear. The heart was of normal size with sounds of good quality; a loud blowing systolic murmur was heard over the precordium, best at the apex. The abdomen was soft and the liver and spleen were not palpable.

Laboratory Tests. Urine: sp. gr. 1015; albumen 0; sugar 0; bile 0; urobilin +; guaiac: negative; microscopic: negative.

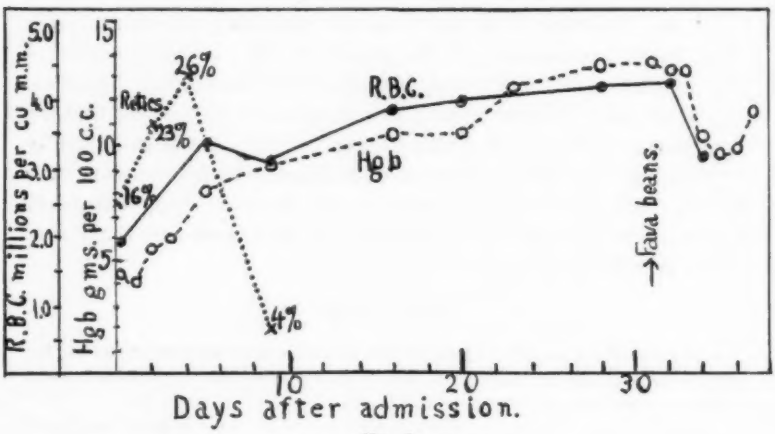

FIG. 1

Blood: Hemoglobin 4.5 grams; R.B.C. 1,900,000; W.B.C. 28,700. Mature neutrophiles 50%, young forms 6%, lymphocytes 40%, monocytes 2%. 1 nucleated red cell per 100 W.B.C. Reticulocytes 16%. The smear showed moderate anisocytosis. Platelets were numerous. A fragility test showed beginning hemolysis at 0.51% NaCl, complete hemolysis at 0.30%, figures which were identical with a normal control.

Course in Hospital. Spontaneous improvement in the patient's condition, which had apparently commenced at the time of admission, continued steadily, the course of the blood changes being indicated in the accompanying chart (fig. 1).

It was evident from the start that the patient was suffering from an acute hemolytic reaction, but there was no evidence of a familial hemolytic dyscrasia, of infection, nor any history of the ingestion of any drug or poison that might account for this. However, on close

questioning as to the food eaten prior to the attack, it was learned that two days before, during the observance of a religious festival, he had eaten a dish of fava beans. Since favism occurs only in certain subjects who are peculiarly susceptible to the beans, it was decided to test the patient's susceptibility by giving him a small dose of beans. One month after admission, when the red cell count had risen to 4 million per cu. mm. and hemoglobin to 13.5 grams, he was given 8 fava beans prepared by his mother by soaking in water without cooking. During the next 3 days no change was noted, but on the fourth day his red count fell abruptly to 3.2 millions and his hemoglobin to 10.2 grams; urobilin appeared in the urine and the stool urobilin increased from 60 mgm. per day to 180 mgm. per day. Skin tests with extracts of the fava bean were negative; this fact does not, however, militate against the diagnosis, for it is usually the case that following an acute attack the skin remains refractory for some time.

The picture presented by our patient is quite typical of that observed in milder cases of favism, as reported in the Italian literature. The disease manifests itself by acute hemolytic crises similar to those seen in other types of hemolytic anemia. In milder cases there is icterus and urobilinuria associated with fever and gastrointestinal symptoms; abdominal or lumbar pain is common, although pain on urination has not been particularly noted. These mild attacks occur within a few days after eating the bean.

More severe attacks are associated with hemoglobinuria and there may be anuria as after a mismatched transfusion. In such cases the symptoms may appear within a few hours and may lead to a fatal termination within two or three days. In cases that recover, the acute symptoms—fever and pain—usually clear within three or four days although the jaundice may last a little longer.

The reduction in the red cell count is usually in proportion to the fall in hemoglobin. The leucocytes and platelets are commonly increased. The recovery phase is initiated by a rise in reticulocytes and in some cases nucleated red cells appear in conspicuous numbers.

It is thus clear that these attacks differ in no way from those seen in the constitutional hemolytic anemias or those due to various exogenous factors—drugs or poisons. Since the specific skin test is usually negative for six or eight weeks following the attack, the only way the diagnosis can be made during this refractory period is from the history

or by the demonstration of hemolysis on eating the bean or exposure to the plant, as was done in the present instance.

The fact that a specific skin test is obtained under certain circumstances would seem to place this condition definitely in the allergic group of diseases that are mediated by specific antigen-antibody reactions. It is of interest that sensitivity to the fava bean tends to be familial and occurs in all degrees of severity. In some instances reported from Sardinia, merely passing a field when the plant is in bloom may cause an attack in a susceptible person. Children are more likely to be susceptible than adults. Racially the disease appears to be largely confined to those who have the characteristics of the old "Mediterranean race" according to Luisada (1). The prevalence in Sardinia where the inhabitants are said to have Mediterranean characteristics especially is attributed to this, for the bean is grown extensively elsewhere in Italy without producing anything approaching the incidence seen on that island. It is of interest that the disease thus appears to have a racial background identical with that of Cooley's anemia. Possibly this peculiar racial background serves to explain a manifestation of allergy that is certainly unique.

SUMMARY

A Greek boy of 5 years was observed in an acute attack of hemolytic anemia which followed the eating of fava beans, his second attack of this nature. Following recovery, a similar attack was induced experimentally.

Attention is called to the fact that the fava bean, although not grown in this country, is frequently imported and eaten by immigrants from Mediterranean countries. Favism should therefore be suspected when hemolytic attacks are observed in persons of Mediterranean origin.

The pathogenesis of the condition is briefly reviewed.

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EXPERIMENTAL STUDIES ON SULFAPYRAZINE IN MICE[1]

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In 1941 Ellingson (1) described the synthesis and the chemical properties of 2-sulfanilamidopyrazine, the para isomer of sulfadiazine, and suggested for it the name of "sulfapyrazine." Publication of this paper was immediately followed by reports (2, 3, 4) of the experimental and clinical use of the compound. Raiziss and his associates (2) found it to be effective in type II pneumococcal infections in mice. Ruegsegger and Schmidt and their coworkers made more extensive studies of the pharmacological and therapeutic properties of sulfapyrazine. They found (3) that doses of 0.5 gram per kilogram, administered parenterally as the sodium salt, were well tolerated by mice but that death usually followed doses of 1.0 gram per kilogram. Later (4) they compared the activity of sulfapyrazine with that of sulfapyridine, sulfathiazole and sulfadiazine against pneumococci *in vitro* and *in vivo*. In the test tube sulfapyrazine appeared to be more active than sulfadiazine and about as active as sulfathiazole and sulfapyridine. In experimental pneumococcal infections in mice, they found that sulfapyrazine was as effective as sulfadiazine and somewhat more effective than sulfapyridine and sulfathiazole. In these therapeutic experiments, equal amounts of the various drugs were given *per os*. The concentrations of sulfapyrazine in the blood were well maintained on the schedule followed, whereas there was considerable fluctuation in the blood concentrations of sulfathiazole and sulfapyridine. Recently (5, 6) their studies were extended to include β-haemolytic streptococci and Friedländer's bacillus. Here again, when equal doses

[1] These studies were supported by a grant from the John and Mary R. Markle Foundation.

We wish to thank the following for the drugs used in this study: American Cyanamid Company for the sulfathiazole; Lederle Laboratories for the sulfadiazine; Mead Johnson and Company for the sulfapyrazine, and the Winthrop Chemical Company for the sulfanilamide.

[2] Dr. Trevett died on May 4, 1944. At the time he became ill, he had completed these studies and prepared a preliminary draft of them for publication. The present manuscript has been revised in minor particulars by Dr. E. A. Bliss.

were employed, sulfapyrazine was found to give results comparable to sulfadiazine and superior to sulfanilamide, sulfapyridine and sulfathiazole.

Marshall and his associates (7) included sulfapyrazine in a study of the activity of a number of sulfanilamide derivatives in infections in mice. Evaluation of the compounds was based on their "median survival blood concentrations" which were then compared to sulfanilamide, as a standard with an activity of unity. By this method of treating the data sulfanilamide, sulfaguanidine and sulfapyridine appear as the most active therapeutic agents against haemolytic streptococci, while sulfapyrazine is one of the least active. On the other hand, sulfapyrazine heads the list in effect upon pneumococcal infections.

Studies have been made on the toxicity, absorption and therapeutic activity of sulfapyrazine in mice. These observations form the basis of the present report.

EXPERIMENTAL PROCEDURES

Materials and Methods

Throughout these experiments the pure CF1 strain of white mice has been used. These animals were maintained on a diet of Wayne Fox Blox. The SV1 strain of type I pneumococcus and the C203 strain of Lancefield's Group A haemolytic streptococcus were used as the test organisms in the therapeutic experiments. The virulence of the cultures was maintained at a maximum by frequent mouse passages and was of such an order that one organism (one colony on a suitably diluted plate) would kill an animal.

Blood levels were determined by the method of Bratton and Marshall (8). The colorimetric readings were made on a Coleman monochromatic spectrophotometer. The red color which develops after the addition of the coupling reagent to the diazotized sulfapyrazine appears promptly and is maintained for at least an hour in the dark. Under these conditions the recovery of both the free and acetylated compounds is incomplete, but at the concentrations employed in these studies the error is insignificant.

Acute Toxicity Studies

Mice of either sex weighing between 19 and 21 grams were selected. The drug was administered subcutaneously in aqueous solution as the

sodium salt, on the basis of an average weight of 20 grams. The animals were allowed no fluid for the 24 hours immediately following the injections, and were observed for a period of 4 days.

Therapeutic Experiments

The method employed is that used in this laboratory and described by Long, Bliss and Ott (9). In the pneumococcal infections sulfapyrazine in a drug-diet concentration of 0.2 per cent was compared to sulfadiazine and sulfathiazole in drug-diet mixtures of 0.125 and 1.0 per cent respectively. In the streptococcal experiments sulfapyrazine 0.2 per cent was contrasted with sulfanilamide in an 0.8 per cent drug-diet mixture. Sulfanilamide was administered in the diet for 4 days and the other compounds for 2 days prior to infection. Animals which ate less than 1 gram of the drug-diet mixture per day were discarded before infection. The blood levels obtained in mice on each of these diets are roughly comparable at the time of infection, averaging between 4 and 7 mgms. per cent. (The figures for sulfanilamide, sulfathiazole and sulfadiazine have been obtained from unpublished observations of Long and Bliss). We found the average blood concentration of 15 mice on an 0.2 per cent diet of sulfapyrazine to be 5.4 mgm. per cent, ranging between 3.3 and 7.5 mgm. per cent. Heart's blood cultures were obtained from representative numbers of mice which died during these experiments. In this way we assured ourselves that most of the deaths which occurred were the result of the experimentally induced infection.

Results

In Table I are recorded the data of our acute toxicity experiments. The LD 50 as calculated by the technique of Reed and Muench (10) is 0.6 gram of sodium sulfapyrazine per kilogram. This figure is to be compared with 1.0 to 1.95 grams per kilogram, found by Long, Haviland and Edwards (11) for sulfapyridine and sulfathiazole, respectively, and 1.5 to 1.75 reported by Feinstone and his associates (12) for sodium sulfadiazine. Accordingly it would appear that sulfapyrazine has a higher acute toxicity than the other compounds.

As may be seen from the accompanying chart (Chart I), the blood concentrations which develop after single oral doses of 0.1 and 0.5 grams per kilogram of sulfapyrazine suspended in gum acacia are not

proportionate to the dosage. It is possible, therefore, that this drug is marginally absorbed from the intestinal tract. It is also to be noted that the concentration of the free drug in the blood reaches a maximum rather promptly but declines very slowly. Significant amounts may

TABLE I

Acute toxicity of sodium sulfapyrazine in mice

DOSE	TOTAL NO. OF MICE	NO. OF MICE DYING	PERCENTAGE DYING
gm./kg.			
0.25	25	7	28
0.50	25	6	24
0.75	25	18	72
1.00	26	24	92
1.50	12	12	100
2.00	13	13	100

remain in the blood for 48 hours. Therefore, the blood concentration-time curve of sulfapyrazine resembles that reported for sulfadiazine (12), and does not have the sharp peak seen after the administration of sulfanilamide (13) and sulfathiazole (14). It is of particular interest

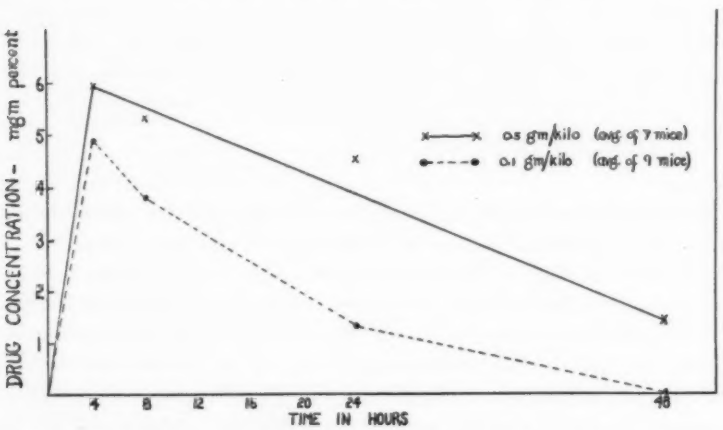

CHART I. ABSORPTION OF SULFAPYRAZINE FOLLOWING SINGLE ORAL DOSES

Blood concentration-time curves of free sulfapyrazine found in mice after single oral doses of 0.1 and 0.5 grams per kilogram suspended in gum acacia.

that the maximal blood levels which develop in mice after single oral doses of sulfapyrazine are only one-third as high as those reported after equivalent doses of sulfadiazine (12).

When mice are allowed to feed on different concentrations of the drug in their diet, it is again apparent (see Chart II), that the blood levels produced are not proportionate to the intake. In addition, it will be seen that the 0.2 per cent drug-diet mixture which we have used in our therapeutic experiments maintains a level of about 8.0 mgm.

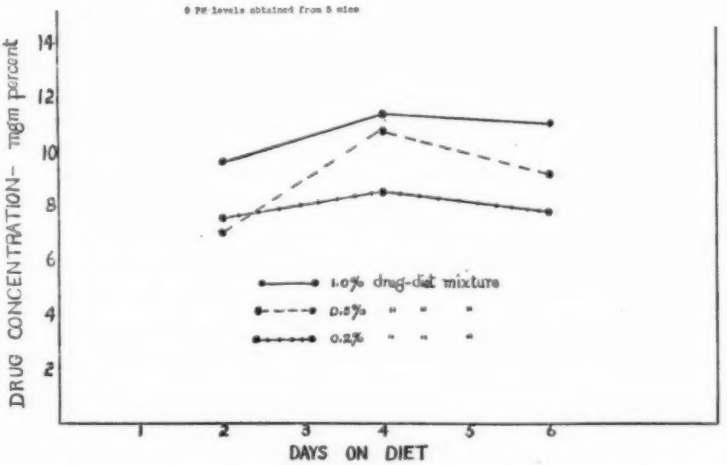

CHART II. ABSORPTION OF SULFAPYRAZINE ADMINISTERED IN DIET

Blood levels of free sulfapyrazine found in mice on various drug-diet mixtures. Each point represents the average of the 9 A.M. and 9 P.M. levels obtained from 5 mice.

per cent. This figure, since it is an average of the 9 A.M. and 9 P.M. blood levels, is significantly higher than that found at the time of infection (9 A.M.), which as stated above, averaged 5.4 mgm. per cent.

As an indication of the amount of conjugation of sulfapyrazine we determined the concentration of free and acetyl sulfapyrazine present in the blood of mice which had been fed on a 0.5 per cent drug-diet mixture for 7 days. In two experiments the grouped bloods of 5 mice gave levels of 9.9 and 13.5 mgm. per cent free, and 13.8 and 18.0 mgm.

per cent total sulfapyrazine. From these observations it is apparent that appreciable amounts of the conjugated compound are present in the blood of mice.

TABLE II

The effect of therapy with sulfanilamide and sulfapyrazine upon the course of experimental haemolytic streptococcal infections in mice with comparable blood levels

DRUG			MICE, NO. USED	BACTERIA, NO. INJECTED	DEATHS (DAYS AFTER INFECTION)																		SURVIVALS	
	Conc. in diet	Avg. daily intake per mouse			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20–25	No.	%
	mgm. %	*mgm. %*																								
S*	0.8	20.6	62	3,000		2		1			4	1	4	1	3	1		2		2				1	40	65
SPz ...	0.2	5.9	56	3,000								2	2	5	3	2	2	1	2		1				36	64
N.....			65	3,000	62	2																			1[t]	1.5

* S = sulfanilamide.
SPz = sulfapyrazine.
N = none (controls).
[t] It is possible that this mouse was not infected.

TABLE III

The effect of therapy with sulfathiazole, sulfadiazine, and sulfapyrazine upon the course of experimental pneumococcal infections in mice with comparable blood levels

DRUG			MICE, NO. USED	BACTERIA, NO. INJECTED	DEATHS—(DAYS AFTER INFECTION)																		SURVIVALS	
	Conc. in diet	Avg. daily intake per mouse			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20–25	No.	%
	mgm. %	*mgm. %*																								
ST*...	1.0	29.0	58	10,000		1		1	1	2	3	3													47	81
SD....	0.125	3.3	58	10,000			3	4	2	4	4	5	2											1	33	57
SPz...	0.2	6.4	60	10,000					1	1	1	3	1												53	88
N.....			59	10,000	2	49	7	1																	0	0

* ST = sulfathiazole.
SD = sulfadiazine.
SPz = sulfapyrazine.
N = none (controls).

Therapeutic Experiments

In these experiments we have compared the therapeutic activity of sulfapyrazine with that of other sulfonamide compounds at comparable

blood levels. Studies now in progress show that there is considerable variation in the blood concentrations of individual mice. However, the average blood concentrations at the time of infection are comparable.

In the experimental streptococcal infections we elected to compare sulfapyrazine with sulfanilamide since the latter drug is known to be one of the most efficacious. As may be seen from Table II, sulfapyrazine gives results as satisfactory as those obtained with sulfanilamide.

In experimental pneumococcal infections, sulfapyrazine was compared with sulfathiazole and sulfadiazine. The highest percentage of survivals (88%) was found with sulfapyrazine as against 81 per cent for sulfathiazole and 57 per cent for sulfadiazine. See Table III.

DISCUSSION

The dose of sodium sulfapyrazine which is fatal to 50 per cent of mice (LD 50) is about 0.6 gram per kilogram of body weight. This is appreciably lower than that of the other sulfonamides now in use, but may have no great clinical significance, since toxic manifestations of sulfonamides in man often appear to be due to sensitivity rather than to overdosage. It is of interest to note that the majority of mice that died succumbed 20 hours after the injection of sulfapyrazine. This is a phenomenon similar to that which appears after treatment with sulfadiazine, and suggests that these animals do not die purely as a result of high concentrations of the drug in the blood. They may succumb from some secondary factor such as renal injury (15). As is the case with sulfadiazine, animals which die remain relatively quiet up to the time of death.

Studies of the chronic toxicity of this drug are incomplete, but have already demonstrated that renal calculi and haematuria may develop in rats which are fed sulfapyrazine.

In our therapeutic experiments sulfapyrazine has proved to be of definite value in the treatment of pneumococcal and haemolytic streptococcal infections in mice. On the basis of blood levels which approximate each other at the time of infection, this compound appears to be as active as sulfanilamide in haemolytic streptococcal infections. In pneumococcal infections sulfapyrazine is about as effective as sulfathiazole and more effective than sulfadiazine.

Although our results differ in certain respects from those reported by Ruegsegger and by Marshall, it is to be noted that the experimental procedure has also differed. These differences emphasize the need for studying chemotherapeutic agents under a variety of experimental conditions before final conclusions as to their efficacy are drawn.

SUMMARY AND CONCLUSIONS

We have studied the absorption, toxicity and therapeutic activity of sulfapyrazine in mice. This compound when administered perorally to mice gives rise to blood concentration-time curves which more closely resemble those seen after the administration of sulfadiazine than those noted when sulfanilamide or sulfathiazole are used. The concentrations of sulfapyrazine attained in the blood are about one-third as high as those observed after equivalent doses of sulfadiazine. They are probably maintained for a longer period of time.

Sulfapyrazine is of definite value in the treatment of haemolytic streptococcal and pneumococcal infections in mice. Its efficacy in haemolytic streptococcal infections appears to be comparable to that of sulfanilamide on the basis of these experiments. Sulfapyrazine is more active than sulfadiazine, and as active as sulfathiazole in the treatment of experimental pneumococcal infections in mice.

Experiments designed to evaluate the acute toxicity of sulfapyrazine for mice indicate that this compound has a greater acute toxicity than sulfanilamide, sulfathiazole and sulfadiazine. However, it is therapeutically very effective in drug-diet concentrations which are not toxic to mice.

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OBSERVATIONS ON A DIET DEFICIENT IN BOTH METHIONINE AND CYSTINE IN MAN*

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The present experiments were undertaken as part of a study designed to throw light on the essential amino acid requirements of man. In a previous report (1) we showed that 3 human subjects on a methionine deficient diet developed negative nitrogen balances which were promptly abolished by the addition of a methionine supplement. These experiments indicated that methionine was a human dietary essential. The role of cystine, however, remained in doubt, for parallel experiments on a cystine deficient diet gave equivocal results in regard to nitrogen balance.

In order to find out whether cystine was dispensable and whether the entire human requirement for sulfur amino acids could be met by methionine we undertook 2 experiments on a double deficiency of methionine and cystine. The attempt was then made to restore the resulting negative nitrogen balance by means of a supplement of methionine alone.

EXPERIMENTAL

Procedure. The subjects, two healthy adult males, were submitted to the following regime. For 6 days prior to the deficiency period a control period was carried out in which both sulfur amino acids were supplied. This was followed by a period of 36 days in which both cystine and methionine were omitted. A supplement of methionine was then given for an additional 6 days.

Diet. As in our previous experiments (2) the basal diet consisted of fats, sugar and certain fruits and vegetables selected for their low nitrogen content. These items contributed approximately 10 per cent

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of the total N intake, the remaining 90% being supplied in the form of a deficient or complete casein digest.

The methionine and cystine deficient digest was prepared as described by one of us (3), and was fed at a level of 0.1 gm. nitrogen per kilo per day. It contained 0.20% methionine and 0.17% cystine. This deficient hydrolysate was supplemented with 3% *dl*-methionine and with 1% *l*(−) cystine in the pre-control periods, and with 3% *dl*-methionine alone in the post-control periods.

TABLE 1

Chemical and morphological changes in the blood of humans in experimental deficiency of of both sulfur amino acids

	SUBJECT 1			SUBJECT 2		
	Control diet	Methionine-cystine deficient diet	Cystine deficient diet (methionine supplement)	Control diet	Methionine-cystine deficient diet	Cystine deficient diet (methionine supplement)
Non-protein-N, gm. %	34.20	25.10	25.90	23.00	28.30	26.00
Total plasma protein, gm. %	7.52	6.78	5.85	5.75	6.90	6.20
Albumin, gm. %	4.35	5.15	4.64	4.40	4.47	4.92
Globulin, gm. %	1.37	1.63	1.21	1.35	1.84	1.28
A/G Ratio	3.17	3.16	3.83	3.25	2.43	3.85
Hemoglobin, gm. %	14.60	16.50	14.80	14.60	15.50	14.70
Red blood cells $\times 10^6$	4.80	5.90	5.00	5.20	4.50	4.60
White blood cells $\times 10^3$	8.00	9.40	7.80	6.70	5.70	6.80

The basal diet supplied 35 to 40 calories per kilo per day and the hydrolysate between 2.6 and 3.5 calories per kilo per day, the over-all caloric intake showing variations from 37.6 to 43.5 calories per kilo per day. The subjects pursued a sedentary life throughout the experiment.

Laboratory Studies. Nitrogen balances were calculated for 6-day periods from Kjeldahl analyses of the food, urine and feces.

Fasting blood samples (10 cc.) were collected in oxalate bottles on the day preceding each change of diet. The total plasma proteins, albumin, globulin, and N.P.N. were determined by a slight modification of the methods described by Robinson, Price and Hogden (4). Hemoglobin was determined with a Klett-Summerson photoelectric

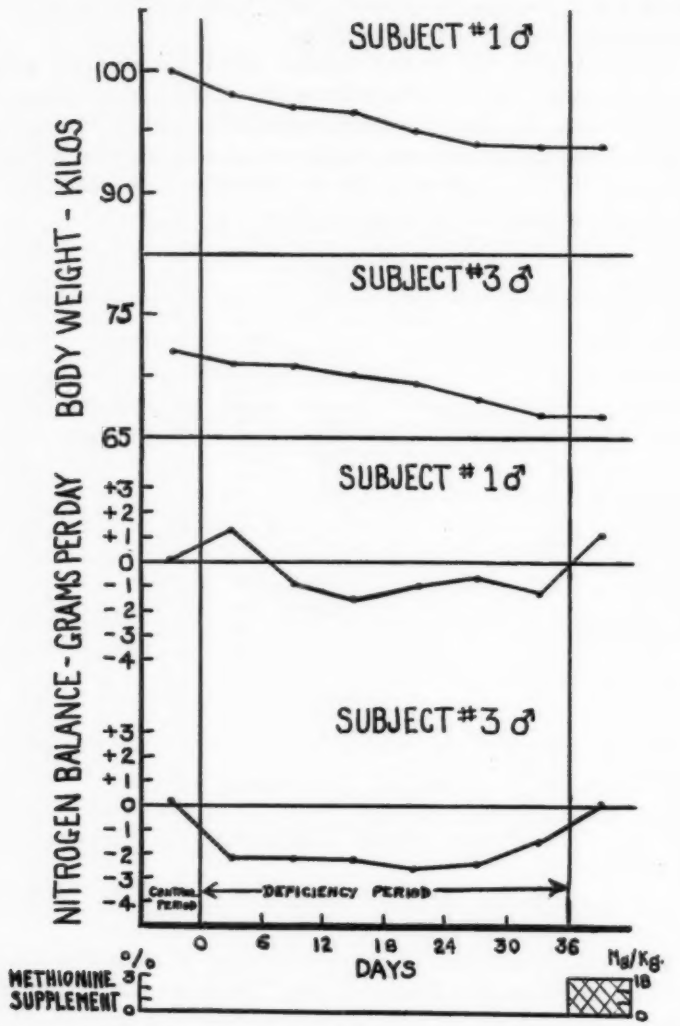

FIG. 1. NITROGEN BALANCE AND WEIGHT CURVES OF HUMAN SUBJECTS ON A DIET DEFICIENT IN METHIONINE AND CYSTINE, SHOWING EFFECT OF METHIONINE SUPPLEMENTATION

colorimeter. Detailed chemical studies of the urine for amino acids and other metabolites were carried out which will be reported elsewhere.

RESULTS

The clinical condition of the subjects was not affected by the deficient diets as far as could be ascertained by subjective symptoms and routine physical examination. Both subjects lost weight during the experiment, as is shown in the accompanying graph. The data from the blood studies are presented in Table 1; they do not reveal any significant changes that may be attributed to the deficiency of the sulfur amino acids.

The data on nitrogen balance presented in figure 1 show that both subjects developed negative nitrogen balances shortly after the institution of the doubly deficient diet and that restoration of nitrogen equilibrium was accomplished in the post-control period by the addition of methionine alone.

These results point to the conclusion that the entire human requirement of sulfur amino acids can be met by methionine alone, a state of affairs which is similar to that which obtains in the rat (5). Our observations are in agreement with those recently described by Rose et al. (6) in a preliminary report; these workers state that they have carried out experiments in man of 6 days duration from which it appeared that omission of methionine from the diet caused negative nitrogen balance, whereas omission of cystine failed to do so.

Our data show that a daily supplement of 18.8 mg. *dl* methionine per kilo will suffice to restore nitrogen equilibrium under the conditions of this experiment. How far below this is the minimal methionine requirement cannot be stated, since supplementation was not carried out in a step-wise manner.

SUMMARY

Two healthy male subjects given a diet deficient in both sulfur amino acids—methionine and cystine—developed negative nitrogen balances. Nitrogen equilibrium was restored by the addition of a supplement of methionine alone, suggesting that methionine is capable of furnishing the entire requirement of man for sulfur amino acids.

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PROCEEDINGS OF THE MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

Held in Hurd Hall, April 10, 1944

Dr. Alfred Blalock, Presiding

1. Studies on Protein Metabolism in Fractures. Dr. John Eager Howard.

SUMMARY

Dr. Howard described metabolism studies on 7 patients with fractures and 5 miscellaneous post-operative cases. Confirming the work of Cuthbertson, he observed a striking loss of nitrogen in the post-fracture cases. This reached its peak about a week after the fractures and then diminished, nitrogen equilibrium being restored in about a month. The post-operative cases showed smaller losses of nitrogen, reaching a maximum on the 3rd or 4th day and lasting only for 9 or 10 days. Various possible factors that might be concerned in producing such nitrogen loss were considered. *Atrophy of disuse* could account for only a small part of the change observed. *Anesthesia* had no effect other than to cause a temporary reduction in food intake. *Drugs* could not be incriminated. *Fever* and *infection* were also considered, but no parallelism could be demonstrated between them and the nitrogen loss. A *reduction in food intake*, although present in some cases, failed to account for the nitrogen loss, nor could this loss be prevented by adequate or even excessive feeding. In one case the administration of an excess of nitrogen parenterally resulted in the excretion of all excess N administered. Studies of potassium balance failed to reveal a loss of K proportional to the loss of N. In fact, in every instance but one, there was a strongly positive potassium balance and this positive potassium balance occurred even during the periods of greatest nitrogen loss.

A final interpretation of these observations is not possible at the present time, but the data raise a number of interesting questions, among which may be mentioned: the possible rôle of adrenal cortical hormones in producing this phenomenon, a view advocated particularly by Albright. One wonders whether this loss of N is beneficial or otherwise, and whether anything is ever gained in such circumstances by forcing nitrogen administration.

DISCUSSION

Dr. Walter Fleischmann made the point that at least two adrenal factors might be concerned—the protein-anabolic N factor and the gluconeogenetic S factor. He called attention to the fact that all Dr. Howard's patients were males and that possibly testosterone might play a rôle. The possibility that the anterior pituitary factors might play a part could not be dismissed, but it was clear that the thyroid was not concerned in the process.

Dr. Henry N. Harkins called attention to the fact that analogous nitrogen losses had been reported in shock and burns; in the latter condition the figures were, however, incomplete, since N losses from the burned surfaces had not been included. He was inclined to attribute the N-loss to internal regulatory factors and pointed out that an increased amino acidemia had been observed by Long after hemorrhage. His own observations on shock pointed to disturbed endocrine factors, notably the hypophysis.

Dr. E. A. Park commented upon the phenomenon of the production of transverse lines in growing bones under various untoward conditions. These transverse lines were caused by an arrest of longitudinal growth in the presence of continuing calcification. He wondered if the formation of a transverse line were not a counterpart of the phenomenon described by Dr. Howard—an evidence of a disturbed nitrogen metabolism.

Dr. Mary V. Buell said that Dr. Howard's experiments afforded a clean-cut demonstration of the deviation of the quantitative relationship of nitrogen and potassium metabolism from that encountered in simple starvation. That the metabolism of these two elements should necessarily run a parallel course is an idea which has lingered in spite of suggestive conflicting evidence of long standing. In 1924, Dr. Harrop demonstrated a reduction of serum potassium under the influence of insulin. During the past decade it has been demonstrated abundantly that the oxidation of many metabolites of diverse chemical nature is coupled with their phosphorylation, a process which takes place inside the cell. In the phosphorylated form, these metabolites are anions which must be covered by some cation. Since potassium is quantitatively the principal cellular cation, it seems logical that it might be retained in larger amounts than normal when the metabolic process is speeded up or takes an abnormal course.

Dr. L. E. Holt, Jr. asked Dr. Howard if he had made any observations on nitrogen partition in the urine. He also wondered if Dr. Howard had any explanation to offer for the discrepancy between his results on the effect of nitrogen forcing and those of Dr. CoTui of New York.

Dr. John Eager Howard, in closing, replied to Dr. Fleischmann that he had no definite information about the excretion of androgens under these conditions, but that if an androgen was concerned, he believed it must be of adrenal origin. He agreed with Dr. Harkins that nitrogen deficit was to be expected in surgical shock and in burns. He was inclined to explain the remarkable retentions of nitrogen reported by Dr. CoTui on forcing nitrogen by the fact that Dr. CoTui's patients were badly undernourished to begin with. Cuthbertson had shown that the losses of nitrogen after fractures occurred only in well nourished rats; the phenomenon could not be duplicated in starved rats.

2. The Naval Medical Corps in Action. CAPT. B. W. HOGAN (M.C.), U. S. N.

Captain Hogan discussed the various medical problems which the Naval Medical Corps had been called upon to solve during the present war—the mechanisms for eliminating the psychiatrically unfit, the problems created by dive bombing

and parachuting, the protection of swimming men from under-water explosions, the desalting of sea water for castaways, the morale problem in submarine warfare. He described the duties of the naval medical officer in the field as exemplified by the Solomon's campaign and gave a vivid account of his experiences during the sinking of the carrier "Wasp."

BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this Journal.)

When Children Ask About Sex, Child Study association of America, Inc., New York, 1943, 16 pp. 20 cents.

This nearly designed pamphlet, which can be read in twenty minutes, should prove very helpful to those mothers and fathers who feel uneasy when their children ask, or fail to ask, questions about sex. The fundamental principle stated is that the best answer is the truth and that the best person to give the answer is the parent. If the parents can attain a frank, wholesome attitude toward sex, and if the children can feel free to come to their parents for the answers, the problem is almost solved. Once the authors have stated the principle, they go on to give sample answers to such questions as: From where does the baby come? What part does the father play? At what age should the child be told about sex? Is masturbation harmful? Is it better for the mother or father to tell an adolescent boy about sex? Should both boys and younger girls be told about menstruation?

The answers given to these questions are simple and clear. Wisely the authors have stated that their answers are only examples. Although the answers are in with the best psychiatric thought of the day, the inadequate ethical foundations and subject matter of some sections will probably make the booklet unacceptable to certain religious groups. However, physicians, teachers, and parents outside of these groups should provide a large and deserved circulation.

A list of books on sex education for children of different ages and for parents and teachers is appended.

R. E. A.

The Permeability of Natural Membranes. By HUGH DAVSON and JAMES FREDERIC DANIELLI. With a Foreword by E. NEWTON HARVEY. 361 pp. Illus. $4.75. Cambridge: At the University Press. New York: The Macmillan Company 1943.

The study of the natural membranes is a basic branch of general physiology. Through quantitative studies on the permeability of l ving membranes information has been obtained both on the structure of the membranes and on the mechanisms whereby cells and organs maintain their chemical composition and their ability to function. The advantages of the red blood cell as an object for the study of cell permeability are emphasized and in accordance with this view the quantitative data obtained by experiments on erythrocytes are presented in great detail. The chapter on the kidney is a short but comprehensive review of the fundamental aspects of glomerular and tubular function. The authors draw attention to the importance of studies using the dual circulation in the kidney of the frog whereby the proximal tubules receive their blood supply from the renal protal vein whilst

the glomeruli receive theirs from the renal branch of the aorta. The problems of membrane structure are discussed with great clarity. The authors have contributed important original work on this problem: Danielli and Davson and Danielli and Harvey have proposed the "paucimolecular" layer theory, according to which the cell membranes consist of a lipoid layer a few fatty molecules thick with an adsorbed protein layer at each fat-water interface. In an appendix the deduction of a fundamental equation for the penetration of a thin membrane of lipoid, not more than 10^{-6} cm. in thickness is presented. The book is well written and is heartily recommended not only to workers in the specialized field of permeability, but to every one interested in general physiology.

W. F.

Maurice Arthus' Philosophy of Scientific Investigation. Translated from the French with an Introduction by HENRY E. SIGERIST. Foreword by WARFIELD T. LONGCOPE. 26 pp., $0.75, The Johns Hopkins Press, 1943.

Dr. Sigerist, in making the translation of this rather inaccessible little masterpiece, and Dr. Longcope, in suggesting that the translation be made and published, have performed a service that should be widely appreciated by those interested in medical and biological investigation. This lucid and inspiring statement of the true investigative spirit and method is one that will always be read with sympathetic understanding by experienced investigators; and, together with Claude Bernard's *Introduction a l'Étude de la Médicine Expérimentale*, it should be read by every person who is beginning, or is contemplating entering, a career of medical investigation. It will be read with hardly less pleasure and with much profit by those who, though themselves not actively engaged in investigative work, are interested in the manner in which science is advanced.

Arthus stresses with clarity the enduring nature of *facts* established by controlled observation, in contrast with the insecurity of *interpretations* of observed phenomena. While this may appear to be a banal and superfluous point, it is one that investigators and readers of their works can hardly hear or think about too often. In forming an opinion about the value, the limits of knowledge, or the significance of any matter, it requires constant vigilance and discipline to keep the fact distinct from the interpretation, and to appreciate clearly the always questionable nature of the latter. The tendency of the mind to allow fact and interpretation to become blurred together, all too often results in a confusion of thought and attitude that can mar not only efforts in science, but also actions of life. Indeed, the methods and morals of science have, in many relations, much to contribute to the art of living.

A. R. R.

Brucellosis in Man and Animals. By I. FOREST HUDDLESON. Revised ed. 379 pp. Illus. $3.50. The Commonwealth Fund, New York, 1943.

This revised book of Dr. I. Forest Huddleson is improved for the clinician by the able discussions of Malta and related fevers in this country and abroad by

the Drs. A. V. Hardy and J. E. Debono, respectively. The author's own particular interests (bacteriologic technique, agglutination and opsonocytophagic tests, and the use of a protein nucleate fraction of brucella organisms in the diagnosis and treatment of brucellosis) are dealt with in detail in separate chapters. A chapter on the eradication or control of sources of brucella infection is contributed by Dr. Ward Giltner, the Dean of Veterinary Division of the Michigan State College. A considerable bibliography is appended.

The detailed considerations of the three brucella species, their morphological characteristics and behavior in culture media and the discussion of selection and preparation of specimens should be of interest to the practising as well as the academic physician. Brucellosis in animals as it occurs in Bang's disease and as it presents itself in the experimental animal is presented with some discussion of gross and microscopic animal pathology.

Altogether the volume is a practical compendium of clinical and laboratory information and methods now in use in the diagnosis and treatment of Brucellosis.

R. N. T.

Atlas of Obstetric Technic. By PAUL TITUS. Illustrations by E. M. Shackelford. 180 pp. $7.00. The C. V. Mosby Company, St. Louis, 1943.

This book undertakes to present in pictorial form, with short titles, the subject of operative obstetric technique. Justification for a volume of this type might conceivably be based either on the unusual distinction of the drawings or on the exceptional teaching value of the text. The present work, in the opinion of the reviewer, fails on both scores. None of the drawings are outstanding; only a few meet modern standards of medical art, while the majority are distinctly crude. Most of the information contained in the legends can be found in any larger textbook of obstetrics.

One rather glaring error is in urgent need of correction. The incision for classical Cesarean section is described as one-third above and two-thirds below the umbilicus. (The illustration shows an incision two-thirds above and one-third below the umbilicus). This, of course, is the old Asa B. Davis incision which most obstetricians abandoned many years ago because it is conducive to a higher incidence of uterine rupture. As may be seen in any of our standard text-books, the incision for classical Cesarean as it is performed nowadays, is wholly below the umbilicus and it seems most regrettable that this older and less desirable technique should be perpetuated.

N. J. E.

Principles and Practice of Obstetrics. By JOSEPH B. DELEE and J. P. GREENHILL. 8th ed. 1101 pp. Illus. $10.00. W. B. Saunders Company, Philadelphia and London, 1943.

Greenhill has preserved the sound principles of obstetrics which have characterized the previous editions of DeLee's textbook. The presentation of the material has been partly rearranged thus offering a more logical continuity in the sequence of events which begin with ovulation and conception, proceed through the

physiology and conduct of pregnancy, and terminate in delivery. New chapters dealing with endocrinology, the use of vitamin K, erythroblastosis, vitamins and minerals, and roentgenotherapy have been added.

The chapter on the toxemias of pregnancy has been entirely rewritten. The section on this complication of pregnancy, which accounts for one-quarter of all maternal deaths, is strangely short in contrast to the lengthy discussions given to certain other conditions, some of which are at best obstetric oddities. There is little mention of hypertensive vascular disease, and very little on the relationship of pre-eclampsia and eclampsia to that complication. Since this textbook is widely circularized among students and general practitioners, the utilization of the chart on page 359 dealing with a case of eclampsia, in which the treatment was "atypical," seems somewhat injudicious.

Latin terminologies have been supplanted by the more widely used English terms. The newer methods in operative technic, such as the Waters' extraperitoneal section, are adequately described. These and other changes have brought the book up to date and it should remain one of the standard sources of obstetric information it has always been.

C. P. M.